Multivalent Thioether–Peptide Conjugates: B Cell Tolerance of an Anti-Peptide Immune Response

David S. Jones,* Stephen M. Coutts, Christina A. Gamino, G. Michael Iverson, Matthew D. Linnik, Martina E. Randow, Huong-Thu Ton-Nu, and Edward J. Victoria

La Jolla Pharmaceutical Company, 6455 Nancy Ridge Drive, San Diego, California 92121.
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Antibodies which bind β_2-glycoprotein I (β_2GPI) are associated with antiphospholipid syndrome. Synthetic peptide mimotopes have been discovered which compete with β_2GPI for binding to selected anti-β_2GPI. A thiol-containing linker was attached to the N-terminus of two cyclic thioether peptide mimotopes, peptides **1a** and **1b**. The resulting peptides, with linker attached, were reacted with two different haloacetylated platforms to prepare four tetravalent peptide-platform conjugates to be tested as B cell toleragens. The linker-containing peptides were reacted with maleimide-derivatized keyhole limpet hemocyanin (KLH) to provide peptide–KLH conjugates. Peptides **1a** and **1b** were also modified by acylation with 3-(4′-hydroxyphenyl)propionic acid *N*-hydroxysuccinimidyl ester. The resulting hydroxyphenyl peptides were radioiodinated and used to measure anti-peptide antibody levels. The KLH conjugates were used to immunize mice to generate an anti-peptide immune response. The immunized mice were treated with the conjugates or saline solution and boosted with the appropriate peptide–KLH conjugate. Three of the four conjugates suppressed the formation of anti-peptide antibody. The stabilities of the conjugates in mouse serum were measured, and the relative stabilities did not correlate with ability to suppress antibody formation.

INTRODUCTION

Multiple copies of a hapten attached covalently to a nonimmunogenic polymeric backbone or platform can tolerize those B lymphocytes producing antibodies that bind the hapten, rendering them nonresponsive to haptenated immunogens (*1*, *2*). The result is a decrease in the amount of antibody produced against the hapten. Molecules such as these, which suppress antibody production by B cells, are called toleragens. For example, 2,4-dinitrophenyl (DNP) groups attached to the D-lysines in polymers consisting of D-amino acids can suppress IgG production in animals which have been immunized with DNP-derivatized proteins. Such findings suggested a therapeutic approach for treatment of autoimmune disorders by specifically tolerizing antigen-specific B cells.

B cell tolerance is the reduction of the ability of a specific B cell to produce antibody. B cell tolerance in vivo can be accomplished by treatment with a toleragen, a molecule bearing multiple copies of hapten. Tolerization of mice, which were immunized with synthetic double stranded oligonucleotides, has been accomplished with a "platform conjugate" consisting of four double-stranded oligonucleotides on a PEG based platform (*3*). This concept was further refined into LJP 394, a molecule designed to treat lupus nephritis (*4*). LJP 394 consists of four double-stranded oligonucleotides connected to a defined platform.

The prospect of developing treatments for other autoimmune diseases has led us to search for peptide mimotopes of naturally occurring antigens. β_2-glycoprotein I (β_2GPI) has been implicated as the natural antigen of what have been historically referred to as anticardiolipin antibodies (ACA), which are associated with arterial and venous thrombosis, recurrent fetal loss, and thrombocytopenia (*5*, *6*). A number of ACA-binding peptides have been discovered by screening random-peptide phage libraries (*7*). Most of these phage-derived ACA-binding peptides are cyclic due to an intramolecular disulfide bond in each. Two analogues of these cyclic peptides have been synthesized as thioether peptide analogues in which the disulfide (S–S) is replaced with a thioether (CH_2–S) (*8*, *9*). Both thioether peptide analogues compete with β_2GPI for ACA which was affinity purified from serum from a single recurrent stroke patient.

The goal of these studies was to develop a method for attaching cyclic thioether peptide analogues to multivalent platforms and test their ability to tolerize an immune response. Thioether peptide analogues were attractive for several reasons. First, a thiol linker can be conveniently used to attach molecules of interest to haloacetylated platforms. A cyclic peptide thioether bond is stable in the presence of a thiol linker, whereas a disulfide bond may be reactive with a thiol linker. Thiol exchange between a thiol and a disulfide can be a rapid reaction leading to mixtures of products (*10*). Second, a thioether bond may be less rapidly metabolized than a disulfide bond (*11*), addressing a major concern with peptide-based drugs.

EXPERIMENTAL PROCEDURES

All chemicals were used as purchased unless otherwise specified. Ethyldiazoacetate, 2-[2-(2-chloroethoxy)ethoxy]-ethanol, $BF_3{\cdot}Et_2O$, thiobenzoic acid, 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), triphenylmethyl chloride, *p*-nitrophenol, dicyclohexylcarbodiimide (DCC), di-*tert*-butyldicarbonate, 3,5-diaminobenzoic acid, *N*-hydroxysuccinimide (NHS), ethylenediamine, triethyleneglycol bischloroformate, and iodoacetic anhydride were purchased from Aldrich Chemical Co. Maleimidyl-activated KLH

* To whom correspondence should be addressed. Phone: (619) 646-6628. Fax: (619) 626-2845. E-mail: dave.jones@ljpc.com.

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Table 1. Analytical Data

compd	mol. formula	type	calcd	found
4	$C_{17}H_{24}N_2O_6$	EA	C, 57.94; H, 6.86; N, 7.95	C, 58.19; H, 6.67; N, 7.77
5	$C_{21}H_{27}N_3O_8$	EA	C, 56.11; H, 6.05; N, 9.35	C, 55.95; H, 5.91; N, 9.12
7	$C_{22}H_{42}N_4O_{10}$	EA	C, 50.55; H, 8.10; N, 10.72	C, 50.42; H, 7.97; N, 10.45
8	$C_{46}H_{70}N_8O_{16}$	EA	C, 55.74; H, 7.12; N, 11.31	C, 55.37; H, 7.09; N, 11.15
3	$C_{34}H_{42}N_8O_{12}I_4$	EA	C, 32.35; H, 3.35; N, 8.87	C, 31.78; H, 3.05; N, 8.32
10	$C_{17}H_{24}O_6SNa$	MS	(M + Na) 379.1191	(M + Na) 379.1203
11	$C_{29}H_{35}O_5S$	MS	(M + H) 495.2205	(M + H) 495.2216
12	$C_{27}H_{30}O_5S$	EA	C, 69.50; H, 6.48	C, 69.33; H, 6.39
	$C_{27}H_{30}O_5SNa$	MS	(M + Na) 489.1712	(M + Na) 489.1723
13	$C_{33}H_{33}NO_7S$	EA	C, 67.44; H, 5.66; N, 2.38	C, 67.56; H, 5.68; N, 2.68
	$C_{33}H_{33}O_7SNa$	MS	(M + Na) 610.1875	(M + Na) 610.1904
15a	$C_{59}H_{101}N_{15}O_{17}S_2$	MS	(M + H) 1357	(M + H) 1357
15b	$C_{59}H_{104}N_{18}O_{17}S_2$	MS	(M + H) 1402	(M + H) 1402
16a	$C_{270}H_{443}N_{68}O_{80}S_8$	MS	(M + H) 6178	(M + H) 6179
16b	$C_{270}H_{455}N_{80}O_{80}S_8$	MS	(M + H) 6359	(M + H) 6361
17a	$C_{284}H_{485}N_{70}O_{82}S_8$	MS	(M + H) 6449	(M + H) 6449
17b	$C_{284}H_{497}N_{82}O_{82}S_8$	MS	(M + H) 6629	(M + H) 6629
20a	$C_{60}H_{96}N_{15}O_{15}S$		(M + H) 1299	(M + H) 1299
20b	$C_{60}H_{99}N_{18}O_{15}S$	MS	(M + H) 1344	(M + H) 1344

was purchased from Pierce (catalog no. 77105). 3-(4′-Hydroxyphenyl)propionic acid *N*-hydroxysuccinimidyl ester (Taggit) was purchased from Novabiochem. Silica gel chromatography was performed on silica gel (230−400 Mesh ASTM) purchased from Baxter. TLC was performed on silica gel TLC plates (5554) manufactured by EM Separations. Phosphate-buffered saline (PBS) was prepared by dissolving 175 g of NaCl, 6.5 g of $NaH_2PO_4 \cdot H_2O$, and 40.9 g of $Na_2HPO_4 \cdot H_2O$ in H_2O and diluting to a final volume of 20 L. Tris-buffered saline (TBS) was prepared by dissolving 219.9 g of NaCl and 151.4 g of tris(hydroxymethyl)aminomethane in H_2O, adjusting the pH to 8.0, at a final volume of 25 L, with HCl.

Melting points are reported uncorrected. NMR spectra were recorded on a Bruker AC-300 spectrometer with broad band probe. Elemental analyses were performed by Desert Analytics of Tucson, Arizona. Mass spectra were recorded on a Finnigan LC-Q electrospray mass spectrometer or obtained from the Mass Spectroscopy Lab at the Scripps Research Institute, San Diego, CA.

Bis-*N*-(tert-butyloxycarbonyl)diaminobenzoic acid, compound 4. A solution of 7.18 g (32.9 mmol) of di-*tert*-butyldicarbonate in 5.5 mL of MeOH was slowly added to a solution of 2.5 g (16.4 mmol) of 3,5-diaminobenzoic acid and 2.76 g (32.9 mmol) of $NaHCO_3$ in 44.5 mL of H_2O and 22.5 mL of MeOH, and the mixture was stirred at room temperature for 24 h. The mixture was cooled to 0 °C, and 6.53 g of citric acid was added. The mixture was extracted with EtOAc, and the combined EtOAc layers were dried ($MgSO_4$), filtered, and concentrated. The residue was dissolved in 40 mL of Et_2O, and the solution was filtered through Celite. The Et_2O layer was extracted with two 40 mL portions of HCl. The Et_2O layer was dried ($MgSO_4$), filtered, and concentrated to give 3.81 g (66%) of **4** as a foamy pink solid; mp 199 °C. ^{1}H NMR ($CDCl_3$): δ 1.55 (s, 18H), 6.80 (brd, 2H), 7.74 (s, 2H), 7.95 (s, 1H) ^{13}C NMR ($CDCl_3$): δ 28.3, 81.4, 113.1, 115.2, 131.1, 139.6, 153.4, 170.7. MS (ESI): $(M + Na)^+$ 375.2. Anal. ($C_{17}H_{24}N_2O_6$) C, H, N (Table 1).

***N*-Hydroxysuccinimidyl Ester of Compound 4, Compound 5.** Dicyclohexylcarbodiimide (3.34 g, 16.2 mmol) was added to a solution of 3.8 g (10.8 mmol) of compound **4** and 1.24 g (10.8 mmol) of *N*-hydroxysuccinimide in 55 mL of EtOAc which had been cooled to 0 °C, and the resulting mixture was stirred for 18 h allowing to come to room temperature. Acetic acid (0.55 mL) was added to the mixture, and the mixture was stirred for 30 min and placed in the freezer at −20 °C for 2 h. The mixture was filtered to remove solids, and the filtrate was concentrated to give 5.80 g of pink foamy solid. Purification by silica gel chromatography (60/40/1 hexane/EtOAc/HOAc) gave 4.30 g (89%) of compound **5** as a slightly pink solid; mp 133−137 °C. ^{1}H NMR ($CDCl_3$): δ 1.55 (s, 18H), 2.94 (s, 4H), 6.81 (s, 2H), 7.79 (s, 2H), 7.96, (s, 1H). ^{13}C NMR ($CDCl_3$): δ 25.6, 28.2, 81.2, 113.9, 114.3, 126.3, 139.8, 152.3, 161.4, 169.0. MS (ESI): $(M + Na^+)$ 472.2. Anal. ($C_{21}H_{27}N_3O_8$) C, H, N.

Mono-*N*-(tert-butyloxycarbonyl)ethylenediamine, Compound 6. This compound was prepared in a manner similar to that described by Huang (*12*). A solution of 1.5 g (25.0 mmol) of ethylenediamine in 15 mL of CH_2Cl_2 was cooled to 0 °C, and a solution of 1.82 g (8.33 mmol) of di-*tert*-butyldicarbonate was added slowly to the mixture. The mixture was stirred at room temperature for 18 h and filtered, and the filtrate was concentrated. Purification by silica gel chromatography (90/10/1 CH_2Cl_2/MeOH/HOAc) gave 0.98 g (67%) of compound **6** as an oil.

Bis-[N_1-(N_2-(*tert*-butyloxycarbonyl)-2-aminoethyl)-carbamoyl]triethylene Glycol, Compound 7. To a solution of 750 mg (4.25 mmol) of compound **6** and 345 μL (337 mg, 4.25 mmol) of pyridine in 6 mL of CH_2Cl_2 was added 445 μL (559 mg, 2.02 mmol) of triethyleneglycol bis-chloroformate. The mixture was stirred for 3.5 h, and partitioned between 35 mL of CH_2Cl_2 and 35 mL of 1 N HCl. The CH_2Cl_2 layer was washed with H_2O, dried (Na_2SO_4), filtered, and concentrated to give 1.14 g of compound **7** as a white solid; mp 105−110 °C. ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 18H), 3.30 (brd s, 8H), 3.68 (s, 4H), 3.72, (s, 4H), 4.24 (s, 4H), 5.16 (brd s, 2H), 5.56 (brd s, 2H). ^{13}C NMR ($CDCl_3$): δ 28.4, 40.5, 41.2, 64.1, 69.6, 70.6, 79.4, 156.3, 156.9. MS (ESI): $(M + Na^+)$ 545.3. Anal. ($C_{22}H_{42}N_4O_{10}$) C, H, N.

Compound 8. Compound **7** (300 mg, 0.57 mmol) was dissolved in 3.5 mL of CH_2Cl_2, and 3.5 mL of trifluoroacetic acid was added. The mixture was stirred for 3 h at room temperature, and the solution was concentrated to give 398 mg of residue. The residue was dissolved in 1.5 mL of H_2O, and to the resulting solution was added 193 mg (2.30 mmol) of $NaHCO_3$ followed by a solution of 567 mg (1.26 mmol) of compound **5** in 6 mL of dioxane. The mixture was stirred for 3 h, acidified with 1 N HCl, and partitioned between 20 mL of 1 N HCl and 30 mL of EtOAc. The aqueous layer was washed with 2 × 20 mL portions of EtOAc. The combined EtOAc layers were washed with saturated $NaHCO_3$ solution, dried ($MgSO_4$), filtered, and concentrated to give 490 mg (86%) of crude compound **8** as a white foamy solid. Purification by silica

gel chromatography (EtOAc) gave 276 mg (48%) of compound **8** as a white foamy solid; mp 138–140 °C. ^{1}H NMR ($CDCl_3$): δ 1.55 (s, 36H), 3.38 (m, 4H), 3.51 (m, 4H), 3.65 (m, 8H), 4.21 (m, 4H), 5.99 (t, 2H), 7.11 (t, 2H), 7.24 (s, 4H), 7.44 (s, 4H), 7.89 (s, 2H). ^{13}C NMR ($CDCl_3$): δ 28.5, 40.6, 41.0, 64.3, 69.8, 70.8, 80.8, 111.6, 112.0, 135.8, 139.7, 153.1, 157.6, 168.4. Anal. ($C_{46}H_{70}N_8O_{16}$) C, H, N.

Compound 3. Compound **8**, (100 mg, 0.1 mmol) was dissolved in 1 mL of 1/1 TFA/CH_2Cl_2, and the mixture was stirred for 1 h at room temperature. Et_2O was added to the mixture to precipitate the resulting tetraamine salt which was dried under vacuum to give a white crystalline solid. The solid was dissolved in 1 mL of H_2O together with 168 mg (2.0 mmol) of $NaHCO_3$, and the resulting mixture was cooled to 0 °C. To the cooled solution was added a solution of 212 mg (0.6 mmol) of iodoacetic anhydride in 2 mL of dioxane. The mixture was stirred at 0 °C for 15 min, acidified to pH 4 with 1 N H_2SO_4, and partitioned between 10 mL of H_2O and 6 × 10 mL portions of 8/2 CH_2Cl_2/MeOH. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated to give 202 mg of yellow oily solid. Purification by silica gel chromatography (9/1 CH_2Cl_2/MeOH) yielded 57 mg (45%) of compound **3** as a white solid; mp 126–140 °C dec.; ^{1}H NMR (1/1 $CDCl_3$/CD_3OD): δ 3.32 (m, 4H), 3.50 (m, 4H), 3.59 (s, 4H), 3.68 (m, 4H), 3.88 (s, 8H), 4.18 (m, 4H), 7.73 (s, 4H), 7.99 (s, 2H), 8.48 (m, amide protons partially exchanged), 10.41 (s, anilide protons partially exhanged). MS (ESI): *m/z* $(M + H)^+$ 1263, $(M + Na)^+$ 1285. Anal. ($C_{34}H_{42}N_8O_{12}I_4$) C, H, N.

Ethyl-11-chloro-3,6,9-trioxaundecanoate, 9. This compound was prepared as described by Boumrah (*13*). A 0 °C solution of ethyldiazoacetate (11.56 mL; 10.704 g; 110 mmol) in 100 mL of CH_2Cl_2 was added dropwise over 30 min to a solution of 2-[2-(2-chloroethoxy)ethoxy]-ethanol (14.54 mL, 16.86 g, 100 mmol) and BF_3·Et_2O (1.23 mL, 1.066 g, 10 mmol) in 150 mL of CH_2Cl_2, under a nitrogen atmosphere, at 0 °C. The solution was stirred for 30 min at 0 °C, and 100 mL of H_2O was added to the reaction mixture. The mixture was transferred to a separatory funnel and shaken vigorously. The CH_2Cl_2 layer was washed twice with 100 mL of H_2O, dried ($MgSO_4$), filtered, and concentrated. The concentrate was distilled under vacuum to give 14.47 g (57%) of **9** as an oil (bp 94–140 °C, 0.25 mmHg). ^{1}H NMR ($CDCl_3$): δ 1.29 (t, 3H), 3.60–3.81 (m, 12H), 4.16 (s, 2H), 4.22 (q, 2H). ^{13}C NMR ($CDCl_3$): δ 14.1, 42.8, 61.0, 68.9, 70.4, 70.4, 70.5, 70.7, 71.2, 170.6.

Ethyl *S*-Benzoyl-11-mercapto-3,6,9-trioxaundecanoate, 10. A solution of 6.51 g (47.1 mmol) of thiobenzoic acid in 25 mL of dry DMF was added to a 0 °C solution of 10.0 g (39.3 mmol) of **9** in 80 mL of dry DMF. To the mixture was added 10.85 g (78.5 mmol) of DBU in 45 mL of dry DMF. The mixture was stirred at 0 °C for 10 min and heated to 70 °C for 16 h. The mixture was concentrated, and the concentrate was partitioned between 200 mL of CH_2Cl_2 and 2 × 200 mL of 1 N HCl. The CH_2Cl_2 layer was washed with saturated $NaHCO_3$, dried ($MgSO_4$), filtered, and concentrated to give 13.2 g of crude product. Purification by silica gel chromatography (7:3 hexane/ethyl acetate) gave 9.49 g (68%) of **10** as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.30 (t, 3H), 3.31 (t, 2H), 3.69–3.78 (m, 10H), 4.18 (s, 2H), 4.23 (q, 2H), 7.48 (m, 2H), 7.59 (m, 1H), 8.00 (d, 2H). ^{13}C NMR ($CDCl_3$): δ 14.4, 28.7, 60.5, 68.4, 70.1, 70.2, 70.4, 70.6, 127.5, 129.0, 133.5, 137.0, 170.5, 191.5. HRMS (FAB) *m/z* calcd for $C_{17}H_{24}O_6SNa$: (M + Na) 379.1191. Found: 379.1203.

Ethyl *S*-Triphenylmethyl-11-mercapto-3,6,9-trioxaundecanoate, 11. A 2.27 M solution of sodium ethoxide in ethanol (10.5 mL, 28.1 mmol) was added under N_2 to a 0 °C solution of 5.0 g (14.0 mmol) of **10** in 15 mL of ethanol, and the mixture was stirred at 0 °C for 30 min. The reaction was acidified to approximately a pH of 2 with 5 N HCl, and the mixture was concentrated. The concentrate was partitioned between 2 × 200 mL of ethyl acetate and 200 mL of 1 N HCl. The combined ethyl acetate layers were dried ($MgSO_4$), filtered, and concentrated. The resulting orange oil was dissolved in 15 mL of CH_2Cl_2, the resulting solution was cooled to 0 °C, and 5.67 mL (5.55 g, 70.15 mmol) of pyridine and 4.30 g (15.43 mmol) of triphenylmethyl chloride was added. The mixture was stirred for 2 h at room temperature and concentrated. The concentrate was partitioned between 3 × 200 mL of ethyl acetate and 200 mL of 1 N HCl. The ethyl acetate layer was dried ($MgSO_4$), filtered, and concentrated. Purification by silica gel chromatography (7:3 hexane/ethyl acetate) gave 4.18 g (60%) of **11** as a viscous oil. ^{1}H NMR ($CDCl_3$): δ 1.28 (t, 3H), 2.46 (t, 2H), 3.32 (t, 2H), 3.47 (m, 2H), 3.60 (m, 2H), 3.69 (m, 2H), 3.73 (m, 2H), 4.14 (s, 2H), 4.21 (q, 2H), 7.20–7.33 (m, 9H), 7.43 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 14.3, 31.9, 61.2, 66.5, 68.7, 69.6, 70.1, 70.3, 70.6, 70.8, 127.1, 128.1, 129.9, 145.0, 170.6. HRMS (FAB) *m/z* calcd for $C_{29}H_{35}O_5S$: (M + H) 495.2205. Found: 495.2216.

***S*-Triphenylmethyl-11-mercapto-3,6,9-trioxaundecanoic acid, 12.** To a solution of 2 g (4.04 mmol) of **11** in 12.2 mL of ethanol was added 1.6 mL (0.648 g; 16.16 mmol) of 10 M NaOH, and the mixture was stirred for 90 min. The mixture was acidified with 1 N HCl to a pH of 2 and partitioned between 200 mL of EtOAc and 200 mL of 1 N HCl. The aqueous layer was washed with two 200 mL portions of EtOAc. The combined EtOAc layers were dried ($MgSO_4$), filtered, and concentrated to give 1.84 g (97%) of **12** as a viscous oil which solidified on standing to give a white solid; mp 59–60 °C. ^{1}H NMR ($CDCl_3$): δ 2.46 (t, 2H), 3.32 (t, 2H), 3.48 (m, 2H), 3.65 (m, 2H), 3.70 (m, 2H), 3.75 (m, 2H), 4.14 (s, 2H), 7.22–7.34 (m, 9H), 7.44 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 31.9, 66.8, 69.6, 69.8, 70.2, 70.4, 71.3, 126.6, 127.8, 129.6, 145.0, 173.2. HRMS (ESI) *m/z* calcd for $C_{27}H_{30}O_5SNa$: (M + Na) 489.1712. Found: 489.1723. Anal. ($C_{27}H_{30}O_5S$) C, H.

4′-Nitrophenyl-*S*-triphenylmethyl-11-mercapto-3,6,9-trioxaundecanoate, 13. To a solution of 1.5 g (3.21 mmol) of **12** in 15 mL of CH_2Cl_2 cooled to 0 °C were added 0.447 g (3.21 mmol) of *p*-nitrophenol and 0.93 g (4.48 mmol) of DCC, and the mixture was stirred at room temperature for 18 h. To the mixture was added 105 μL (0.108 g; 1.79 mmol) of acetic acid, and stirring was continued at room temperature for 1 h. The mixture was placed in the freezer for 30 min and filtered. The filtrate was concentrated, and the residue was purified by silica gel chromatography (70/30/1 hexane/EtOAc/HOAc) to give 1.65 g (87%) of **13** as white crystals; mp 73–74 °C. ^{1}H NMR ($CDCl_3$): δ 2.47 (t, 2H), 3.35 (t, 2H), 3.48 (m, 2H), 3.62 (m, 2H), 3.75 (m, 2H), 3.85 (m, 2H), 4.47 (s, 2H), 7.20–7.35 (m, 11H), 7.42 (m, 6H), 8.29 (d, 2H). ^{13}C NMR ($CDCl_3$): δ 32.0, 66.6, 68.5, 69.6, 70.1, 70.4, 70.8, 71.2, 122.2, 125.2, 126.7, 127.9, 129.6, 145.0, 155.0, 168.5. HRMS (FAB) *m/z* calcd for $C_{33}H_{33}O_7SNa$: (M + Na) 610.1875. Found: 610.1904. Anal. ($C_{33}H_{33}NO_7S$) C, H, N.

Attachment of Linker 13 to 1a, Synthesis of Compound 15a. To 15 mg (0.013 mmol) of compound **1a** were added 160 μL of a solution of 29.5 mg of compound **13** and 17.5 μL of diisopropylethylamine in 0.5 mL of DMF. The mixture was stirred for 2 h and

Table 2. Reduction of Anti-Peptide Antibodies in Mice[a] Treated with Compounds 16a, 16b, 17a, and 17b as Determined by the Farr Assay

	experiment 1 (treatment with 16a)[b]			experiment 2 (treatment with 16b)[b]			experiment 3 (treatment with 17a)[b]			experiment 4 (treatment with 17b)[b]		
dose (nmol)	mean	SD	reduction	mean	SD	reduction	mean	SD	reduction	mean	SD	reduction
none	203	72		275	188		231	61		275	188	
1.25							298	59	0%			
2.5				59	31	78%	204	94	11%	19	13	93%
5.0	100	71	51%	112	42	41%	192	103	17%	109	62	60%
10.0	82	16	59%	44	16	84%	213	93	8%	52	46	81%
50.0	65	36	68%	73	66	73%	295	127	0%	104	68	62%

[a] Five mice per group were immunized with peptide–KLH conjugate **19a** or **19b**. Three weeks later, the mice were treated with tetravalent peptide conjugate. Five days later, the mice were boosted with peptide–KLH conjugate. Seven days later, the mice were bled. [b] Antigen-binding capacity (ABC) (see refs *15* and *16*). [c] ABC significantly lower than control (experiment 1, $p = 0.0157$; experiment 2, $p = 0.0015$; experiment 4, $p = 0.0015$).

precipitated by addition of Et_2O. The precipitate, compound **14a**, was dried under vacuum and dissolved in 650 μL of a solution of 1/1/0.056/0.040 TFA/CH_2Cl_2/thiophenol/Me_2S, and the solution was allowed to stand for 1 h. Precipitation by addition of Et_2O gave crude compound **15a** which was purified by HPLC (C18, 15–45% CH_3CN/H_2O 0.1% TFA). Fractions containing pure product were lyophilized to give 8.6 mg (48%) of **15a** as a white solid; MS (ESI): *m/z* (M + H) 1357.

Attachment of Linker 13 to 1b, Synthesis of Compound 15b. To 100 mg (0.084 mmol) of compound **1b** was added 1.05 mL of a solution of 88.5 mg of compound **13** and 52.5 μL of diisopropylethylamine in 1.5 mL of DMF. The mixture was stirred for 16 h and precipitated by addition of Et_2O. The precipitate, compound **14b**, was dried under vacuum and dissolved in 3.35 mL of a solution of 1/1/0.056/0.040 TFA/CH_2Cl_2/thiophenol/Me_2S, and the solution was allowed to stand for 2 h. Precipitation by addition of Et_2O gave crude compound **15b** which was purified by HPLC (C18, 15–45% CH_3CN/H_2O 0.1% TFA). Fractions containing pure product were lyophilized to give 60 mg (51%) of **15b** as a white solid; MS (ESI): *m/z* (M + H) 1402.

Conjugate 16a. To a solution of 45 mg (33.2×10^{-6} mol) of **15a** in 3.32 mL of He-sparged pH 8.5, 200 mM borate buffer was added 1.02 mL of a 8.2 mg/mL solution of **3** in MeOH. The mixture was stirred for 16 h and 12 mL of 10% HOAc/H_2O solution was added. The mixture was purified by HPLC (C18, gradient 30–45% CH_3CN/H_2O 0.1% TFA) to give 9.0 mg of **16a**; MS (ESI) *m/z* calcd for $C_{270}H_{443}N_{68}O_{80}S_8$: (M + H) 6178. Found: 6179.

Conjugate 16b. To a solution of 33.1 mg (23.6×10^{-6} mol) of **15b** in 2.8 mL of He-sparged pH 8.5, 100 mM borate buffer was added 707 μL of a 8.4 mg/mL solution of **3** in MeOH. After 2 h, another 6.6 mg of **15b** was added. The mixture was stirred for 3 h and 0.5 mL of 10% HOAc/H_2O solution was added. The mixture was purified by HPLC (C18, gradient 30 to 40% CH_3CN/H_2O 0.1% TFA) to give 23.0 mg of **16b** MS (ESI) *m/z* calcd for $C_{270}H_{455}N_{80}O_{80}S_8$: (M + H) 6359. Found: 6361.

Conjugate 17a. To a solution of 8.6 mg (6.3×10^{-6} mol) of **15a** in 630 μL of He-sparged, pH 8.5, 200 mM borate buffer was added 40 μL of a 40 mg/mL solution of **2** in 9/1 MeOH/H_2O. The mixture was stirred for 24 h, and 1 mL of 10% HOAc/H_2O solution was added. The mixture was purified by HPLC (C18, gradient 25 to 55% CH_3CN/H_2O 0.1% TFA) to give 8.3 mg of **17a**. MS (ESI) *m/z* calcd for $C_{284}H_{485}N_{70}O_{82}S_8$: (M + H) 6449. Found: 6449.

Conjugate 17b. To a solution of 38.0 mg (27.1×10^{-6} mol) of **15b** in 2.7 mL of He-sparged, pH 8.5, 100 mM borate buffer was added 452 μL of a 13.5 mg/mL solution of **2** in MeOH. The mixture was stirred for 2 h and 0.5 mL of 10% HOAc/H_2O solution was added. The mixture was purified by HPLC (C18, gradient 15 to 35% CH_3CN/H_2O 0.1% TFA) to give 20.0 mg of **17b**; MS (ESI) *m/z* calcd for $C_{284}H_{497}N_{82}O_{82}S_8$: (M + H) 6629. Found: 6629.

Preparation of KLH Conjugate, 19a. Compound **15a** (2.5 mg, 1.8 μmol) was dissolved in 0.43 mL of PBS containing 1 mM EDTA, and to the resulting solution was added 0.43 mL of a 10 mg/mL solution of maleimidyl-activated KLH (Pierce Chemical catalog no. 77105) in water. The mixture was tumbled slowly for 2h at room temperature and was exhaustively dialyzed against PBS at 4 °C. The presence of conjugated peptide was demonstrated by the binding of relevant ACA to microplates coated with KLH–peptide conjugate (*14*).

Preparation of KLH Conjugate, 19b. The preparation of **19b** was done essentially similarly to the preparation of **19a** using **15b** instead of **15a**.

Preparation of HOP Peptide, 20a. Compound **20a** was prepared essentially similarly to the preparation of **20b**, using **1a** instead of **1b**, to provide **20a** as a white solid. MS (ESI): *m/z* $(M + H)^+$ 1299.

Preparation of HOP Peptide, 20b. To a solution of 10 mg (8.4 μmol) of peptide **1b** in 840 μL of pH 8.5 200 mM sodium borate buffer was added 110 μL (41.8 μmol) of a solution of a 100 mg/mL solution of 3-(4′-hydroxyphenyl)propionic acid *N*-hydroxysuccinimidyl ester in DMSO. After 2 h, 10 mL of 8/1/1 H_2O/CH_3CN/HOAc was added, and the resulting solution was loaded onto a preparative HPLC column and purified by HPLC (C_{18}, 12 mL/min, gradient 8/2/0.1 H_2O/CH_3CN/TFA to 6/4/0.1 H_2O/CH_3CN/TFA over 40 min, 210 nm, retention time 26–30 min) to provide 6 mg of **20b** as a white solid after lyophilization. MS (ESI): *m/z* $(M + H)^+$ 1343.

In Vivo Biology. Mice (C57Bl/6, five mice per group) were immunized with **19a** or **19b**, a conjugate of the peptide covalently attached to KLH. The immunizations were accomplished by intraperitoneal (IP) injection of 0.5 mL of a solution containing 10 μg of the conjugate (**19a** or **19b**) precipitated on 2 mg of alum and 2×10^9 *Bordetella pertussis* organisms as an adjuvant (*15*). Three weeks after immunization, the mice were injected, IP, with the test toleragens **16a**, **16b**, **17a**, or **17b** at the doses indicated in Table 2. Mice immunized with **19a** were injected with **16a** or **17a**, while mice immunized with **19b** were injected with **16b** or **17b**. A control group was injected with 0.5 mL of saline instead of the toleragen. Five days later, mice in both groups were boosted with the peptide–KLH conjugate in saline solution. Seven days later, all of the mice were bled, and serum was harvested. The anti-peptide antibody levels of the serum, reported as antigen-binding capacity (ABC), was determined by a Farr assay where the radioactive antigen **20a** or **20b** was present at 10^{-8} M (*15*, *16*).

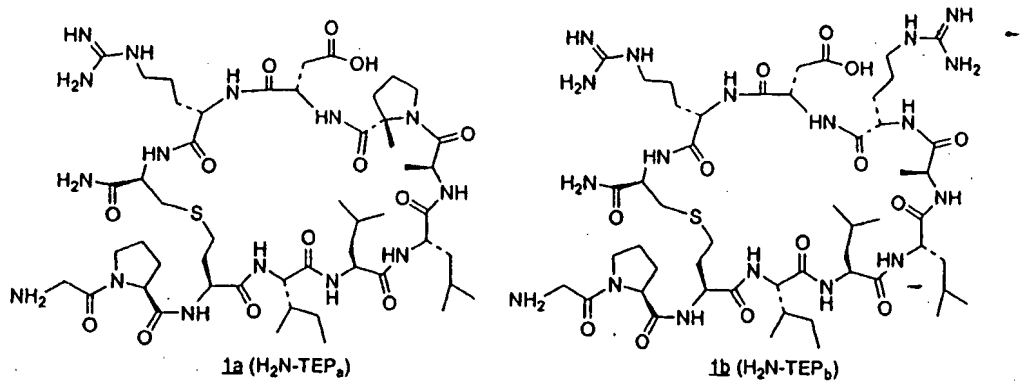


Figure 1.

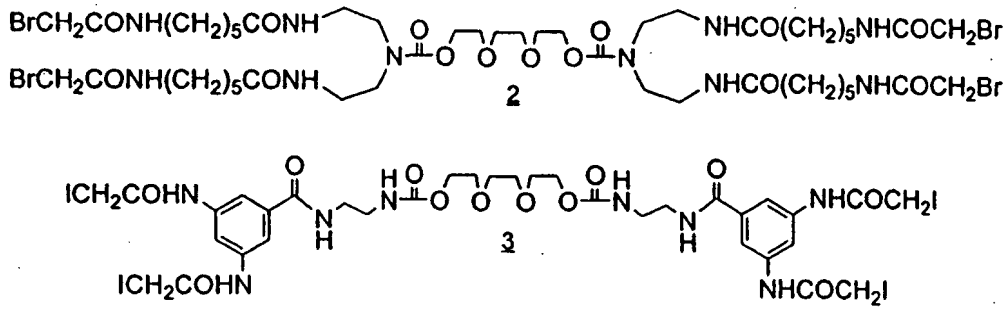


Figure 2.

Determination of Serum Levels of Anti-Peptide Antibodies Using the Farr Assay. Hydroxyphenylated peptide, **20a** or **20b**, was trace radiolabeled as follows. To 50 μg of hydroxyphenylated peptide in 50 μL of, pH 7.5, 100 mM phosphate buffer (PB) was added 1 mCi of ^{125}I (New England Nuclear, NE2–033H) followed by 25 μL of a solution of 2 mg/mL of chloramine T in PB. After 1.5 min, a solution of 25 μL of a solution of 2 mg/mL of sodium metabisulfite in PB was added. The mixture was loaded onto a Sep-pak C_{18} cartridge (Waters part no. 020515), which had been washed with CH_3CN followed of H_2O. The column was eluted with H_2O to wash away salts, then with 50/50 CH_3CN/H_2O to elute peptide. Radiolabeled peptide fractions were pooled and used as radioactive antigen in the Farr assay (*15*). Serum samples from the mice were diluted by factors of 6, 36, and 216 with 10% normal rabbit serum in 0.1 M, pH 8.6, borate buffer, and the radiolabeled peptide was added to the mixture to give a peptide concentration of 10^{-8} M. The IgG was precipitated by addition of an equal volume of saturated ammonium sulfate solution, and the counts in the precipitate were measured to determine the percentage of counts bound to the IgG. The values are reported in Table 2 as antigen-binding capacity (ABC) (*16*).

Serum Stability Studies. To 720 μL of mouse serum (Sigma) was added 80 μL of a 10 mg/mL solution of conjugate (**16a**, **16b**, **17a**, or **17b**) in water, and the mixture was incubated at 37 °C. For each time point, 100 μL of the mixture was added to 900 μL of a cocktail which contained **1b** as an internal standard (80 μL of a 10 mg/mL solution of **1b** in water added to 7.12 mL of a 1 M solution of guanidine HCl in 99/1 water/TFA). The resulting solution was loaded onto a C_{18} Extract Clean column (Alltech Associates Inc., part no. 205 000, 100 mg/1.0 mL) which had been washed with 2 mL of MeOH and 4 mL of water. The peptide conjugate and internal standard were eluted with 1 mL of MeOH, and the eluate was filtered and analyzed by HPLC (C_{18}, gradient 15/85/0.1 CH_3CN/H_2O/TFA to 30/70/0.1 CH_3CN/H_2O/TFA, 0 to 10 min, then 30/70/0.1 CH_3CN/H_2O/TFA to 35/65/0.1 CH_3CN/H_2O/TFA, 10 to 20 min).

DISCUSSION AND RESULTS

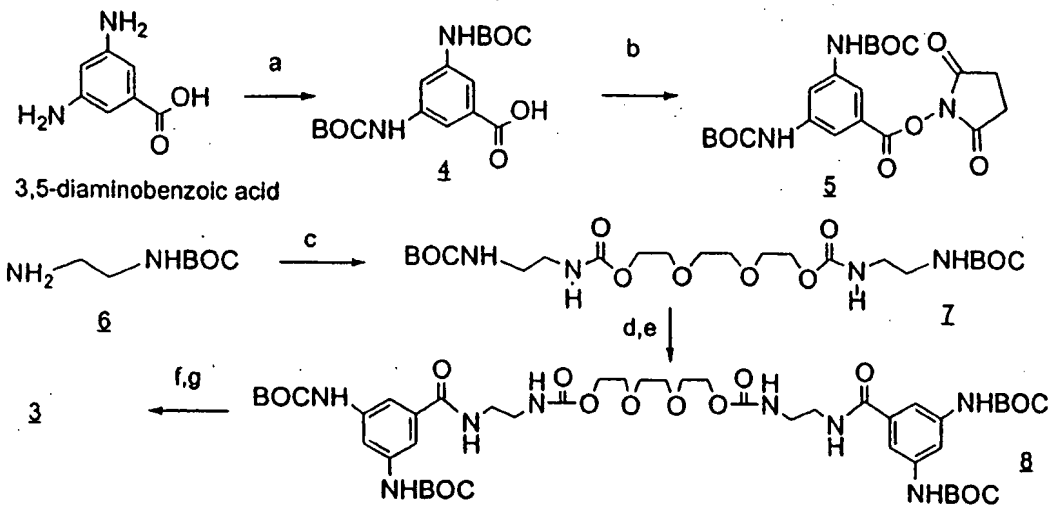
To test the ability of cyclic thioether peptides **1a** and **1b** to effect tolerance when presented on a multivalent platform, chemistry was developed to prepare both tolerageneic and immunogenic conjugates.

Peptides have been conjugated to a variety of substances including other peptides, proteins, and carrier molecules. Some examples of the various methods used are mentioned below. Each method has its own advantages and disadvantages, and the best method for a particular application depends on a number of factors such as what functional groups are present on each partner in the coupling reaction and the solvent compatibility of the partners.

Condensation of protected peptide fragments with amino poly(ethylene glycol) (amino-PEG) has provided PEG–peptide conjugates (*17*). Carboxy-terminal cysteines have been used to link peptides to PEG via disulfide bonds (*18*). Peptide segments have been ligated to provide native proteins by taking advantage of the ability of an N-terminal cysteine on one peptide to react with C-terminal thioesters on another peptide to give a thioester which can rearrange to a natural amide bond (*19*). Ligation via thiazolidine formation between a C-terminal aldehyde and an N-terminal cysteine is another method of connecting two unprotected peptides (*20*). For a review of protein synthesis by chemical ligation see Walker (*21*).

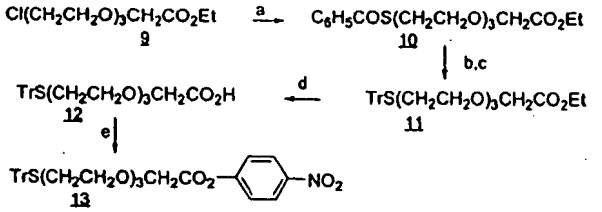
Bromoacetylated peptides have been attached to antibodies (*22*) or ligated to a second cysteine-containing peptide (*23*) via thioether bonds. Peptides with cysteine sulfhydryl groups have been attached to DNA using thioether bonds (*24*). Another method of attaching peptides to other biomolecules is oxime formation between aminooxy peptides and aldehyde or ketone groups (*25*).

Scheme 1[a]



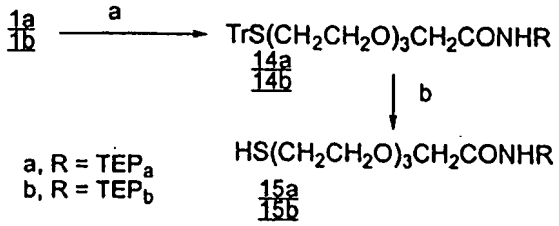
[a] (a) $(BOC)_2O$, MeOH, H_2O, $NaHCO_3$ (66%); (b) NHS, DCC, EtOAc (89%); (c) triethyleneglycol bis-chloroformate, pyridine, CH_2Cl_2 (quantitative); (d) TFA/CH_2Cl_2; (e) compound *5*, $NaHCO_3$, dioxane, H_2O (48%, two steps); (f) TFA/CH_2Cl_2; (g) Iodoacetic anhydride, $NaHCO_3$, dioxane, H_2O (45%, two steps).

Scheme 2[a]



[a] (a) Thiobenzoic acid, DBU,DMF (58%); (b) NaOEt, ethanol; (c) trityl chloride, pyridine, CH_2Cl_2 (50%, two steps); (d) NaOH, ethanol (98%); (e) *p*-nitrophenol, DCC, CH_2Cl_2 (87%).

Scheme 3[a]



[a] (a) Compound **13**, DIPEA, DMF; (b) 1/1/0.056/0.04 TFA/CH_2Cl_2/thiophenol/Me_2S (48%, both steps).

Reductive amination of peptide amino groups with oxidized dextran has been used to immobilize peptides on ELISA plates (*26*).

In this report, multivalent thioether peptide conjugates were prepared by first attaching a thiol-containing linker to the thioether peptides, then using the thiol to form a thioether bond with a multivalent haloacetyl platform molecule. This provides a tetravalent conjugate consisting of four identical cyclic thioether peptides attached to a common nonimmunogenic carrier platform. The objective of preparing such conjugates was to test their ability to tolerize an anti-peptide immune response in a model in which mice were immunized with peptide attached to keyhole limpet hemocyanin (KLH).

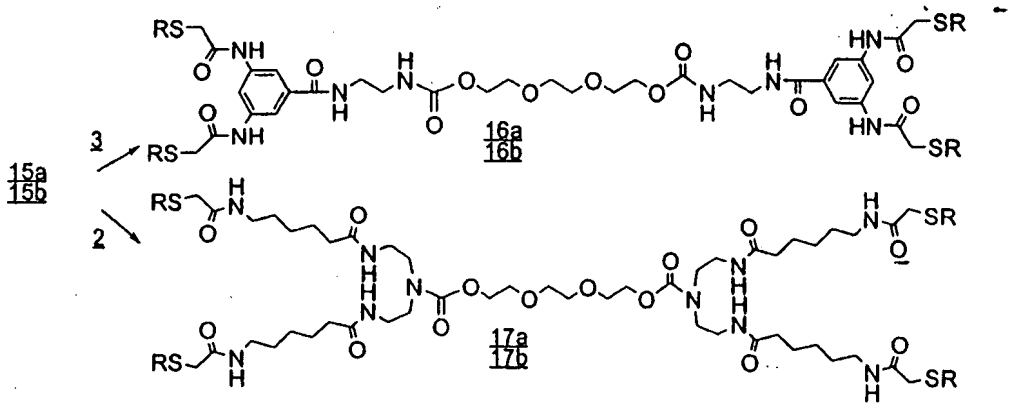
Cyclic thioether peptide analogues **1a** (H_2N-TEP_a) and **1b** (H_2N-TEP_b), which compete with β_2GPI for selected ACA are shown in Figure 1. The sequences of these peptides were derived from phage libraries which contained two fixed cysteines with seven variable amino acids between the cysteines (*27*). Phage which bound ACA were sequenced, and the sequences were used to prepare peptides which bound ACA when oxidized to cyclic disulfides. Peptides **1a** and **1b** are analogues of the primary sequences in which the disulfide bond is replaced with a thioether bridge. In addition peptide **1a** contains an α-methyl proline replacement of a sequence which originally contained a proline at that position. Incorporation of α-methyl proline resulted in tighter binding to ACA and is expected to confer protease resistance.

Peptides **1a** and **1b** were prepared by two different methods for preparing cyclic thioether analogues of cyclic disulfide peptides. Compound **1a** was prepared, as previously described, using a presynthesized thioether intermediate, which was incorporated in solid-phase synthesis (*8*). This method used an amide bond formation to affect cyclization. Compound **1b** was prepared by an alternate method which involved cyclization of a cysteine sulfhydryl with a 4-chloro-2-amino-butyric acid residue which was incorporated in solid-phase synthesis (*9*). The only difference between **1a** and **1b**, is in the fourth amino acid from the C-terminus. Compound **1a** has an (*S*)-α-methylproline at that position, whereas compound **1b** has an arginine at that position. The α-methylproline analogue was included in this study, because it was anticipated that it would have greater metabolic stability. Both analogues have similar ACA binding properties. The relative molar concentrations for 50% inhibition (IC_{50}) of binding of ACA to β_2GPI for **1a** and **1b** are 1.32×10^{-4} M and 2.17×10^{-4} M (*28*).

Tetravalent conjugates of both cyclic thioether peptide analogues, **1a** and **1b**, were prepared using two platforms, compounds **2** and **3**, which are shown in Figure 2. Compound **2** was available from previous studies (*4*). Compound **3** was designed as a hybrid of **2** and a poly(ethylene glycol)-based platform (*3*) which had been previously used successfully in earlier tolerance studies with melittin peptides (*29*).

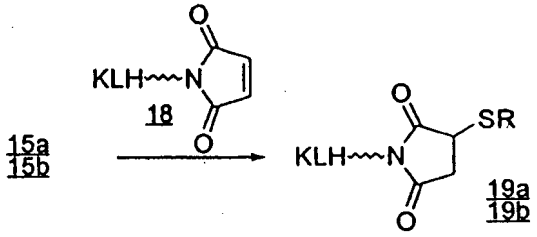
The synthesis of compound **3** is described in Scheme 1. The amino groups of 3,5-diaminobenzoic acid were protected with *tert*-butyloxycarbonyl (BOC) groups to provide compound **4**. Treatment of **4** with *N*-hydroxysuccinimide in the presence of DCC gave the NHS ester, **5**. Mono-BOC-protected ethylenediamine, **6**, (*12*) was condensed with triethylene glycol bis-chloroformate to provide **7**. The BOC groups were removed, and the resulting

Scheme 4[a]



[a] a, R = $(CH_2CH_2O)_3CH_2CONH\text{-}TEP_a$; b, R = $(CH_2CH_2O)_3CH_2CONH\text{-}TEP_b$.

Scheme 5[a]



[a] a, R = $(CH_2CH_2O)_3CH_2CONH\text{-}TEP_a$; b, R = $(CH_2CH_2O)_3CH_2CONH\text{-}TEP_b$.

diamine was condensed with **5** to provide **8**. Finally, the BOC groups were removed and the resulting amines were acylated with iodoacetic anhydride to provide **3**.

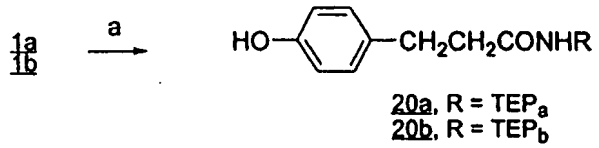
A trityl protected thiol linker, compound **13**, was developed which could be attached to the N-terminus of **1a** or **1b** via acylation. This linker was designed to provide some distance between the platform and the peptide, and to provide water solubility. The synthesis of **13** is diagramed in Scheme 2. Chlorotriethylene glycol was converted to compound **9** by a modification of an existing procedure (*13*). Treatment of **9** with thiobenzoic acid provided the thioester **10**. The thioester was removed by treatment with sodium ethoxide, and the resulting thiol was protected by tritylation to give trityl-thioether **11**. The ester was saponified with sodium hydroxide to provide the acid **12**, which was esterified with DCC and *p*-nitrophenol to provide **13**.

The linker was attached to thioether peptides **1a** ($H_2N\text{-}TEP_a$) and **1b** ($H_2N\text{-}TEP_b$) by acylation at the N-terminus by reaction with **13** as shown in Scheme 3. The resulting peptide products with linker attached, **14a** and **14b**, were treated with trifluoroacetic acid in the presence of scavengers to remove the trityl group and provide **15a** and **15b**, the peptides with the free thiol linker.

Tetravalent conjugates were prepared as described in Scheme 4. Compound **15a** was reacted with platform **3** in pH 8.5 aqueous sodium borate to provide **16a**. Reaction of **15a** with **2** in the same buffer gave conjugate **17a**. Compound **15b** reacted with **3** to give **16b** and with **2** to give **17b**. The reaction mixtures, containing crude conjugates, were loaded directly onto a preparative HPLC column for purification by preparative HPLC.

KLH conjugates **19a** and **19b** were prepared for the purpose of immunizing and boosting mice with peptide. The KLH conjugates were prepared as follows and as described in Scheme 5. A solution of **15a** or **15b** in PBS was added to an aqueous solution of KLH which was modified with maleimide groups on lysine ϵ-amines

Scheme 6[a]



[a] (a) 3-(4′-Hydroxyphenyl)propionic acid *N*-hydroxy succinimidyl ester, DMSO, pH 8.5, borate.

(compound **18**, Pierce Chemical Co.) resulting in the formation of putative KLH peptide conjugates **19a** and **19b**. These conjugates were not chemically characterized, since KLH itself is an ill-defined multisubunit-containing protein; however, they were characterized by their ability to bind ACA when coated on ELISA plates. The conjugates were also characterized functionally by their ability to elicit an anti-peptide immune response in mice.

Hydroxyphenylated peptides, **21a** and **21b**, were prepared as follows for use in the Farr assay, a solution phase assay for determining antigen binding capacity (ABC) (*15*). Thus, peptides **1a** and **1b** were hydroxyphenylated by adding a solution of 3-(4′-hydroxyphenyl)-propionic acid N-hydroxysuccinimidyl ester (Taggit, Novabiochem) in DMSO to a solution of peptide in pH 8.5 borate buffer to provide **20a** and **20b** as described in Scheme 6.

The Farr assay is a radioimmunoprecipitation assay which measures the amount of radiolabled antigen found in immunoglubulin which has been precipitated in the presence of antigen. The Farr assay is our method of choice for measuring antibody levels when the unbound antigen does not precipitate under the antibody-precipitating conditions. It is our method of choice because it is a relatively simple assay to run, it is reproducible, and it gives a numerical result, the antigen-binding capacity (ABC), which is independent of the dilution at which the assay is run.

An immunization protocol was designed to specifically produce antibodies against the peptide. These anti-peptide antibodies would not exist in the absence of the immunization, because the peptide is totally synthetic. Thus, the ability of compounds **16a**, **16b**, **17a**, and **17b** to tolerate an anti-peptide immune response was tested in mice which were immunized with the relevant peptides conjugated to KLH, conjugates **19a** and **19b**. Three weeks later the mice were treated with one of the tetravalent peptide conjugates, **16a**, **16b**, **17a**, or **17b**. Five days after treatment with the tetravalent peptide conjugates, the mice were boosted with a second administration of the relevant peptide conjugated to KLH (**19a** or **19b**). Seven days after boosting, the ABC of treated and control mice

Table 3. Degradation of Conjugates 16a, 16b, 17a, and 17b in Mouse Serum

compd	time to 50% degradation (h)
16a	10.7
16b	3.6
17a	13.3
17b	3.0

were measured using a Farr assay at dilutions of 1/6, 1/36, and 1/216 (*15*). These data are summarized in Table 2. The specificity of the treatments was demonstrated with measurements of both anti-peptide antibody titers using the Farr assay, and anti-carrier (KLH) antibodies using an ELISA assay (*30*). Three of the conjugates, **16a**, **16b**, and **17b**, resulted in a significant reduction in the anti-peptide antibody levels, whereas, there were no reductions in the anti-KLH antibody levels in any of the experiments (data not shown). Thus, these toleragens were specific for downregulating the anti-peptide response without affecting the response to the protein carrier, KLH. Conjugate **17a** did not reduce the anti-peptide response at the doses used, perhaps due to differences in the distribution, clearance, or metabolic properties of **17a** from the other conjugates. The stabilities of the conjugates in serum were studied to determine if ability to tolerize correlated to metabolic stability. The results, shown in Table 3, indicate that compound **17a** is actually the most stable conjugate of the four conjugates tested in normal mouse serum, so its lack of tolerance activity cannot be explained by the issue of susceptibility to serum proteases.

These studies show that cyclic thioether peptide antigen mimotopes, when presented as a tetravalent conjugate, can cause downregulation of an anti-peptide antibody response. The downregulation is specific to the antibodies against the immunizing peptide as evidenced by no downregulation of antibodies against the carrier protein, KLH, which was used in the immunization. The downregulation is evidence of B cell tolerance, since the tetravalent conjugate is administered 5 days before the boost, at which time little or no toleragen would be present.

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(16) The percentage of antigen bound multiplied by the dilution factor is the same as the antigen binding capacity (ABC) up to 35% bound, because the percentage of antigen bound is directly proportional to dilution in that range. For bindings greater than 35%, the log of ABC is proportional to the log of the percentage bound and is calculated by the following formula [ABC = [2 × 1/10 antilog(% bound/35 × log 3.5)] × dilution factor]. Values of ABC reported are the average of ABC values calculated from percentage of antigen bound in the range 10–70% (*15*).

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